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Radiolytic Effects of Gamma Rays on α -Amylase in Aqueous Solution

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In order to investigate the effects of irradiation on the peptide bonds of this enzyme protein, search for small peptides by gel filtration, measurement of molecular weight by ultracentrifugal analysis and determination of N-terminal amino acid by the DNP method were carried out in the intact enzyme and irradiated one. From the results obtained from these experiments, it was concluded that the peptide bonds did not accept any cleavage by the irradiation.

Irradiation of γ -rays on α -amylase in aqueous solution was found to reduce the amount of several amino acid residues to some extent, such as lysine, histidine, arginine, tyrosine and so on. Free ammonia were liberated from the enzyme protein by the irradiation of γ -rays. The degree of liberation of the free ammonia was proportional to that of inactivation and was independent of the enzyme concentration at the irradiation. It was assumed that the liberation of the ammonia was due to the destruction on the basic amino acid residues in this protein molecule. Inactivation of α -amylase by irradiation may be carried out through the specific damage of amino acid residues which consist in the active center or have great influence upon it.

INTRODUCTION

In the previous paper¹⁾, it was reported that the inactivation of *Bacillus subtilis* α -amylase in aqueous solution by γ -ray irradiation was caused by the indirect action of radiation, in other words, by the reaction between the solute, α -amylase, and the radiolyzates of the solvent water. It was also found that hydroxyl radicals were more effective to inactivation of the enzyme than other radiolyzates of water.

At the beginning of investigation of the mechanism of enzyme inactivation by irradiation, experiments concerning the action of radiation on the peptide bonds of enzyme protein were carried out. In order to investigate wheather or not the peptide bonds accept cleavage by irradiation, gel filtration for the search of small peptide, ultracentrifugal analysis for the measurement of molecular weight and the DNP method for the determination of N-terminal amino acid were tried and there results will be described in the present paper. However, α -amylase irradiated in aqueous solution and reduced its activity has not accept any damage in its peptide chain. In order to investigate the inactivation mechanism of α -amylase by γ -ray irradiation, secondly was studied the effects of irradiation on the amino acid residues of side chain in this enzyme protein. From this point of view, the experiments treating of the change in the amino acid composition of intact and irradiated α -amylase and the

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liberation of free ammonia by irradiation were carried out and the results will be described in this paper.

MATERIALS AND METHODS

Bacillus subtilis α -amylase used in this work was a crystalline preparation made by Daiwa Kasei Co. Ltd. from the culture of *Bacillus subtilis* DT-57 strain. To remove the contaminated impurities and stabilizer, the enzyme solution was filtrated through a column of Sephadex G-25. Concentration of the enzyme protein in the solution was estimated from the absorption at 280 m μ assuming $E_{1\text{cm}}^{1\%}=27.0$. For the conversion of gravity to molarity, a molecular weight of this enzyme was assumed to be 48,500, according to the results from the ultracentrifugational analysis described here.

Molecular weight of the enzyme protein was determined by the Archibald's method²⁾ with a Hitachi Analytical Ultracentrifuge Apparatus UCA-1 type. An 0.64 per cent α -amylase solution in a 0.1 M sodium acetate-0.08 M NaCl-0.005 M CaCl₂ buffer pH 6.9, ionic strength 0.2, was equilibrated by a dialysis versus the same buffer for 24 hrs at 5°C and then centrifuged at a speed setting of 9,690 r.p.m. at 16°C. The exposures were taken with a diagonal slit of 80°, 20 min, 40 min, 60 min and 80 min after the rotor had reached full speed. Specific gravity was measured by using a Ostwald picnometer, capacity 1 ml.

γ -Ray irradiation was carried out using the 2000 Ci ⁶⁰Co Irradiation Facility with the dose rate of 1.2×10^5 rad/hr in the Institute for Chemical Research, Kyoto University, and the Toshiba's RE 1010 100 Ci ⁶⁰Co Irradiation Facility³⁾ with the dose rate of 4.63×10^4 rad/hr.

Amino acid composition of the enzyme was determined by the method of Stein and Moore⁴⁾ with a KLA-2 Hitachi amino acid analyzer. Standard specimens of amino acids used in this determination were of a synthetic pure product of Tanabe Pharmaceutical Chemical Co. Ltd.. In order to discriminate inconsiderable difference of the amino acid composition between non-irradiated and irradiated enzyme, the degree of hydrolysis for various times, 16, 22, 48 and 76 hours, were measured in the non irradiated enzyme and in the irradiated one respectively, according to the method of Stein and Moore. The values obtained from the amino acid analysis were collected by the data from these measurements. Quantitative analysis of tryptophan residue was made by the method of ultra violet light absorption⁵⁾ separately.

The species of N-terminal amino acid was determined by the Sanger's DNP method⁶⁾.

The free ammonia blended in the enzyme protein was isolated and measured by the Conway's microdiffusion method⁷⁾ with some improvement as follows: 1 ml of borate-NaOH buffer, pH 11.5, was added into 1-2 ml of protein solution included ammonia in a bottle for diffusion, and immediately a rubber stopper with a glass rod immersed in 5N-H₂SO₄ was putted on the bottle, which was rotated at 30°C for 1.5 hours. The diffused ammonia was adsorbed in the H₂SO₄ of the glass rod, washed out into a test tube with 3 ml of distilled water, and then was measured colorimetrically by the method of indophenol⁸⁾ using ammonium sulfate as standard substance.

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Hydrolysis of amides in the enzyme protein was carried out through the two processes, one was an acid hydrolysis by 3N-HCl, and the other was an alkali hydrolysis by saturated NaOH. In the alkali hydrolysis, the mixture of 1 ml of 0.2 percent α -amylase solution and 1 ml of saturated NaOH in the same bottle as described in METHODS, was rotated at 32°C for 2.5 hours. The hydrolyzed and liberated ammonia was adsorbed in 5 N-H₂SO₄ and measured colorimetrically as described above. In the acid hydrolysis, the mixture of 1 ml of 0.3 percent of α -amylase solution and 1 ml of 6 N-HCl and several drops of octanol was heated at 110°C for 3 hours in a test tube with a refrigerator. This hydrolyzate was removed into the diffusion bottle and after the addition of 1 ml of saturated NaOH, the ammonia in that was isolated and measured in the same manner.

RESULTS

Gel filtration

Gel filtration was applied using a 2×30 cm column packed with Sephadex G-75, for non irradiated and irradiated α -amylase solution. The result is shown in Fig. 1. The remaining activity ratio of the enzyme irradiated with 7.8×10^4 rad was about 50 percent. The elution patterns and the retention volumes were almost similar respectively between non irradiated enzyme and irradiated one. This data indicates that the enzyme did not undergo a change on its molecular size by the irradiation.

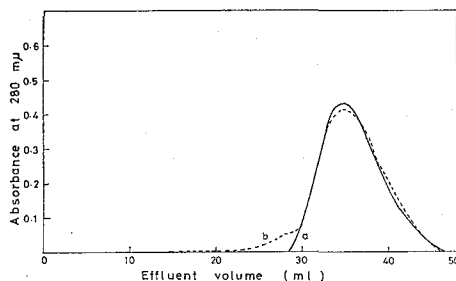


Fig. 1. Reduction of amino acid residues by irradiation. Moles of amino acid residues reduced per enzyme protein molecule was shown against the inactivation ratio by the irradiation. Dose irradiated in each points was identical with that of Table I.

Measurement of molecular weight

An alteration of the molecular weight of α -amylase by irradiation was measured according to the Archibald's method. Each molecular weight shown in Table I is an average of every measurements obtained from the data in the various sedimentation equilibrium runs. It had been reported by Fischer *et al.*⁹⁾ or Isemura *et al.*^{10,11)} that two molecules of α -amylase of *Bacillus subtilis* with a molecular weight of about 45,000 associate as a dimer bound by a Zn²⁺ ion usually. According to Fischer *et al.*, the dimer dissociates into two monomers in the presence of metal-binding agents such as calcium salt at ethylenediamine tetra-acetic acid (Ca-EDTA), hydroxyquinoline, citrate and oxalate *etc.* On the one hand, according to Isemura *et al.* the monomer-

Table I. Molecular weight of non irradiated and irradiated enzyme protein calculated by the Archibald's method.

	Mm	Mb	Survival rate of Enzyme activity
none irradiated	97,890	96,882	100%
irradiated by 5.9×10^4 rad	96,903	98,211	50%

Mm: molecular weight calculated at the meniscus

Mb: molecular weight calculated at the bottom of cell

dimer transformation depends upon the concentration of α -amylase and the separation of the dimer with a sedimentation coefficient of 6S and the monomer with that of 4.5S can be carried out through a gel filtration of Sephadex G-100. Under the conditions in the present work, α -amylase should be considered to exist as a dimer. Therefore, the molecular weight as a dimer was estimated about at 48,500. The centrifuge pattern and the calculated molecular weight of non irradiated enzyme and those of irradiated one were essentially identical. Either any alteration of the molecular weight or dissociation of dimer to monomer was not found from this result.

Determination of N-terminal amino acid

The determination of N-terminal amino acid of the irradiated and non irradiated α -amylase was carried out by the method of Sanger. As shown in Table II, N-terminal amino acid of both enzymes was found to be valine alone. The results obtained by gel filtration and ultracentrifugation described above, denied the alteration in the molecular weight of this enzyme protein. However, they cannot deny the possibility of cleavage or isolation of small peptides and amino acids.

Table II. N-terminal amino acid of non irradiated and irradiated enzyme protein. (by Sanger's DNP method)

irradiation Dose	none	4.6×10^4 rad	5.5×10^4 rad	4.6×10^5 rad
Survival rate of enz. Activity	100%	65%	50%	20%
neutral acidic	Valine	Valine	Valine	Valine
basic ϵ -Lysine	ϵ -Lysine	ϵ -Lysine	ϵ -Lysine	ϵ -Lysine
ϵ -Lysine	ϵ -Lysine	ϵ -Lysine	ϵ -Lysine	ϵ -Lysine
di-Lysine	none	none	none	none

From the fact that any new species of N-terminal amino acid was not found in the irradiated enzyme, it was concluded that the peptide main chain had not accept any cleavage by irradiation.

The amino acid composition of intact and irradiated α -amylase was measured by the method of Stein and Moore. Since the degree of hydrolysis of the irradiated enzyme is more or less different from that of non-irradiated enzyme, the values shown in Table III have been collected by the data obtained from the experiments done in the variation of hydrolytic time. For example, in the intact α -amylase, the amounts of aspartic acid, glycine, alanine, threonine, valine, *etc.* were reached in their maximum value after a hydrolysis as long as 48 hours, but both in the irradiated protein

Radiolytic Effects of Gamma rays on α -Amylase in Aqueous SolutionTable III. Amino acid composition of non-irradiated and irradiated α -amylase.

Amino Acid	Dose	none	5.9×10^4 rad	9.8×10^5 rad	data by Junge et al. ¹²⁾	data by Akabori et al. ¹³⁾
Aspartic acid		54.7	55.0	53.3	53	54.6
Threonine		21.5	22.0	20.0	23	25.7
Serine		23.5	21.0	23.4	24	28.6
Glutamic acid		42.7	38.2	41.7	43	44.1
Proline		16.1	15.0	14.2	14	17.7
Glycine		37.5	36.2	34.8	39	36.2
Alanine		28.5	27.7	27.0	29	32.6
Cystine		—	—	—	—	—
Valine		23.8	23.7	23.7	25	22.9
Methionine		5.1	4.6	4.7	5	4.1
Isoleucine		16.2	16.5	15.0	17	14.6
Leucine		21.5	21.7	20.2	23	23.6
Tyrosine		23.5	23.2	20.8	24	22.1
Phenylalanine		17.1	15.7	15.3	18	17.1
Tryptophan		14.1*	14.3*		15	14.6
Lysine		24.8	26.2	20.2	25	24.1
Histidine		11.4	12.5	9.35	12	11.8
Ammonia		66.7	—	—	—	37.5
Arginine		17.3	18.0	15.3	17	18.7

The amounts of each amino acid is described as number of amino acid residues per enzyme molecule.

Molecular weight of α -amylase used in this data is 48,000. Inactivation rates of enzyme to irradiation of each dose are,

5.9×10^4 rad: 30 percent.

9.8×10^5 rad: 95 percent.

remaining 70 percent of its original activity and the irradiated one remaining only 5 percent, a hydrolysis of 22 hours was found to be satisfactory for the complete hydrolysis in almost all amino acids. The results obtained in these manner, were shown in Table III.

Since the result from the analysis of the intact α -amylase were intermediate between the result of Junge *et al.*¹²⁾ and that of Akabori *et al.*¹³⁾, this result was seemed to be reasonable one considering the difference in the strain of *Bacillus Subtilis*. In the chromatograms of this analysis any unidentified peak was not found even in the irradiated enzymes. The amino acid compositions of both the enzyme irradiated with 5.9×10^4 rad, inactivated 30 percent and that irradiated with 9.8×10^5 rad, inactivated 95 percent, were not so different from that of non irradiated enzyme on the whole. However, the amounts of basic amino acids, such as lysine, histidine and arginine, and tyrosine were found to be reduced a little as the irradiation dose increased. In this irradiated enzyme which are inactivated to 10 percent of its original activity, it has lost 4.5 moles of lysine, 2 moles of histidine and arginine, and 2.7 moles of tyrosine in one enzyme molecule.

The amounts of amide in this α -amylase were measured by the method described in METHODS. Intact α -amylase was found to include 36 moles of amide in its mole-

cule.

Liberation of ammonia by irradiation

0.13 percent, 0.34 percent and 0.69 percent α -amylase solutions were irradiated with various doses of γ -rays and the amounts of ammonia liberated from the irradiated protein were measured by the method described in METHODS. Figure 2 shows the number of ammonia liberated, versus the dose irradiated and versus the inactivation ratio of the enzyme. The amount of free ammonia in each solution increased with the irradiation dose and as shown in Fig. 2, the degree of liberation of the free ammonia was found to proceed proportionally to that of inactivation by irradiation. Furthermore this finding was independent of the enzyme concentration at the irradiation. In the irradiated enzyme which was completely inactivated, 1.5-1.7 moles of ammonia were removed per 1 molecule of the enzyme.

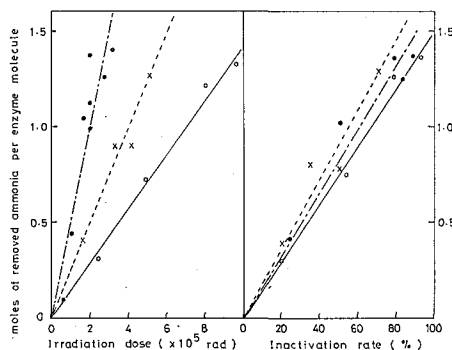


Fig. 2. Liberation of ammonia from enzyme protein by irradiation. Moles of removed ammonia per enzyme molecule is shown against irradiated dose and against the inactivation ratio.

(●): The concentration of enzyme solution at the irradiation is 1.3 mg/ml.,
(×): 3.4 mg/ml., (○): 6.9 mg/ml.

DISCUSSION

Although the amino acid composition of α -amylase was not found to be altered so largely by irradiation, the amounts of tyrosine and several basic amino acids, such as lysine, histidine and arginine, were reduced to some extent as described above. These amino acid residues are well known to be comparatively sensitive to irradiation¹⁴⁾, but it is not clear whether or not they play important parts to reveal the enzyme activity. In the case of this α -amylase, the importance of these residues for this enzyme activity have been suggested from the studies using the methods modifying these amino acid residues by specific chemical reagents. Therefore, the reduction of these amino acid residues by irradiation is likely to be caused by the damage of active sites in this enzyme. On the contrary, considering the high sensitivity of these amino acids to irradiation, it is also assumed that these reduction may be only an accompanying result with the destruction of protein conformation. These problems should be discussed formally standing on the investigations treating with the conformation changes by irradiation elsewhere. As shown in Fig. 2 the free ammonia

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was liberated by irradiation. Since it was cleared that the peptide bond of α -amylase was not cleaved by irradiation the free ammonia should originate not in the NH_2 of the peptide bonds nor of the N-terminal amino acid, but in the deamination of acid amides or basic amino acid residues in side chains. The results from the amino acid analysis showed a little reduction of basic amino acids and acid amides such as glutamine or asparagine were inclined to be resistant to irradiation in its single solution. These facts seemed to suggest that the ammonia was the result from the destruction of basic amino acids, but cannot deny the possibility of the destruction in acid amides.

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